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Genomic sequence analysis of the United States infectious laryngotracheitis vaccine strains chicken embryo origin (CEO) and tissue culture origin (TCO)

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ABSTRACT

The genomic sequences of low and high passages of the United States infectious laryngotracheitis (ILT) vaccine strains CEO and TCO were determined using hybrid next generation sequencing in order to define genomic changes associated with attenuation and reversion to virulence. Phylogenetic analysis of available full genomes grouped strains into three major clades: TCO, CEO, and Australian. Comparative genomics revealed that TCO attenuation is likely the result of an ORF C truncation. Genes involved in attenuation are generally clade-specific, however four genes ORF C, U_L27, U_L28 and U_L39 commonly contained various mutations across the CEO and TCO lineages. The Thr644 mutation in the U_L27 gene encoding glycoprotein B was identified in all virulent US strains. The U_S10 gene was identified as a potential virulence factor for the TCO revertant 81658. The U_L41 gene was responsible for the robust gain in virulence of CEO-Fowl Laryngotracheitis® after 20 passages in chickens.

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Introduction

Infectious laryngotracheitis (ILT) is a highly contagious respiratory disease of chickens caused by *Gallid herpesvirus-1* (GaHV-1) (Guy and Garcia, 2008), a member in the genus *Iltovirus, of the Alphaherpesvirinae* subfamily within the family *Herpesviridae* (Davison et al., 2005a). First described by May and Tittsler (1925), the severe form of the disease is characterized by clinical signs including, watery eyes, hemorrhagic conjunctivitis, nasal discharge, respiratory rales, gasping, marked dyspnea, and expectoration of blood-stained mucous. Morbidity and mortality can vary depending on the viral strain, and the severe epizootic form of the disease can cause morbidity up to 100% and mortality as high as 70% (Guy and Bagust, 2003). Although it was the first poultry pathogen controlled by vaccination, ILT is still a major problem in areas where dense bird populations exist (Bagust and Johnson, 1995). Over the last 40 years the disease has largely been controlled through mass vaccination with live attenuated strains derived from virulent US field strains circulating in the late 1950s and early 1960s (Guy and Garcia, 2008). These isolates were attenuated through serial passage either in embryonated eggs (CEO) or in embryonic chicken cell cultures (TCO) (Gelenczei and Marty, 1962; Samberg and Aronovici, 1969). There are four commercially available CEO vaccines in the US. Three of these vaccines, Trachivax®, LT Blen® and Fowl Laryngotracheitis® originated from the Hudson strain, a virulent field isolate that dates back to Beaudette and Hudson (1933). The fourth is Laryngo-Vac® which originated from the Cover strain (Cover and Benton, 1958). Only one TCO product LT-Ivax® is commercially available and originated from a virulent field strain isolated in the early 1950s (Gelenczei and Marty, 1962).

In addition to their abilities to induce protective immunity and prevent clinical signs and mortality (Fulton et al., 2000; Han and Kim, 2003), all live attenuated GaHV-1 vaccines can persist in a latent state in apparently healthy birds (Andreasen et al., 1989; Hughes et al., 1989). Long-lived birds are therefore reservoirs of vaccine viruses that can potentially be reactivated and contribute to the spread of the disease (Andreasen et al., 1989; Hilbink et al., 1987; Rodríguez-Avila et al., 2007). Live attenuated vaccines can also easily transmit from vaccinated to unvaccinated birds, and after sequential passages in birds they may regain virulence. Recombinant viral vectored vaccines expressing GaHV-1 genes encoding glycoproteins (e.g. fowlpox expressing glycoprotein B and Melagrid herpesvirus expressing glycoproteins D and I) were introduced in 2007 as safer GaHV-1 vaccine alternatives (Cochran and MacDonald, 1998; Tong et al., 2001).

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Despite similarities in their protective indices, some differences do exist among the CEO and TCO vaccine strains. Rodríguez-Avila et al. (2007) reported that the rates of replication and transmission of the CEO vaccine strain are greater than those of the TCO vaccine. Although both vaccine strains can regain virulence after bird-to-bird passage, CEO revertants cause a more severe respiratory disease with higher mortality rates, when compared to TCO revertants at the same bird passage level (Brandly and Bushnell, 1934; Guy et al., 1991). In spite of shortcomings associated with reversion to virulence most US commercial layers and broiler breeders, particularly those raised in locations with high exposure risk, are vaccinated with either TCO vaccine applied by the eye-drop route or with CEO vaccine applied in drinking water or by coarse spray. However, broilers are vaccinated with CEO vaccines only in an imminent outbreak (Davison et al., 2005b). Although vaccination with the live-attenuated ILT vaccines has been widely utilized in breeders and layers, severe and long-lasting outbreaks in broilers have been frequently reported (Davison et al., 2005b).

Early molecular epidemiology studies involving restriction endonuclease (R.E.) profiling have demonstrated that the majority of the isolates associated with outbreaks in the US showed R.E. banding patterns closely resembling those of digested CEO DNA, while outbreaks related to TCO-like isolates were fairly rare (Guy et al., 1989; Keeler et al., 1993; Keller et al., 1992). To date, differentiation of vaccine strains from virulent field isolates has mainly involved restriction fragment length polymorphisms of PCR products (multilocus PCR-RFLP analyses) (Creelan et al., 2006; Kirkpatrick et al., 2006; Neff et al., 2008; Oldoni et al., 2008) and limited nucleotide sequence analysis (Han and Kim, 2001; Neff et al., 2008). Using multi-locus PCR-RFLP analyses, (Oldoni et al. (2008)) have reported that US isolates from commercial and non-commercial poultry can be genotyped into nine clades (I through IX). Over a decade (1997 to 2007), this procedure grouped 68 commercial poultry outbreak-related isolates into four genotypes (groups III, IV, V, and VI). Seventy six percent (52/68) were genotyped as either similar to CEO vaccine Group IV or the closely related Group V genotype. Only three isolates were genotyped as closely related to the TCO vaccine (Group III genotype). The remaining 19% (13/68) isolates appear to be dissimilar to the CEO and TCO genotypes and belong to the virulent group VI genotype (Oldoni and Garcia, 2007; Oldoni et al., 2008). In a European study of isolates collected over 35 years, 94% (98/104) of the isolates were also shown to be related to ILT vaccine strains (Neff et al., 2008). Similarly, by determining the nucleotide sequence of ICP4 amplicons, Sadeghi et al. (2011) reported that Iranian GaHV-1 field isolates were phylogenetically indistinguishable from CEO vaccine strains. Based on these data, it has been postulated that North American, European and Middle Eastern CEO vaccine strains have reverted to virulence and that these revertants have become established in the poultry populations (Ojkic et al., 2006).

The genomic changes that determine reversion to virulence and attenuation of CEO- and TCO-related isolates are largely unknown and full genome deep sequencing analysis of related strains may provide some insight. Although DNA sequencing data from GaHV-1 has been publicly available since the late 1980s, the first complete genome was assembled by Thureen and Keeler (2006) from partial genome sequences of different strains obtained in six laboratories across the globe. Recently, non-composite full-length genome sequences of the European CEO vaccine strain Serva (Lee et al., 2011b), two Australian vaccine strains (A20 and SA2) (Lee et al., 2011a), two Australian virulent strains CL9 and ACC78 (Lee et al., 2012), two US CEO strains (LT Blen® and Laryngo-Vac®) (Chandra et al., 2012) and four virulent US isolates (Spatz et al., 2012) (USDA reference challenge strain [Group I], broiler breeder isolates 81658 [Group III], 63140 [Group V] and 1874C5 [Group VI] were obtained. The objective of this study was to identify genomic changes associated with reversion to virulence and attenuation of GaHV-1 live attenuated vaccines strains by comparing full genome sequences of current CEO Trachivax® and TCO LT-Ivax® vaccines (Merck Animal Health, Summit, N.J. USA) with chicken passages 1 and 20 of CEO Fowl Laryngotracheitis® and TCO vaccine LT-Ivax® performed in 1991 by Guy et al. (1991), as well as sequences of known virulent US GaHV-1 isolates.

Results and discussion

Origin of the virus isolates

Guy et al. (1991) conducted an experiment in which Fowl Laryngotracheitis® (CEO) and LT-Ivax® (TCO) vaccines were passaged twenty consecutive times in chickens after initial intra-tracheal inoculation. Following virus isolation and plaque purification, stocks of the 1st (p1) and 20th (p20) passages of the CEO Fowl Laryngotracheitis® and the TCO LT-Ivax® vaccines were propagated in the chorioallantoic membrane of chicken embryos and stored in the repository of Dr. Jim Guy at North Carolina State University (Raleigh, N.C.). The origin, genotypes, and passage history of vaccine strains and virulent isolates are presented in Table 1. Since additional in vitro passages were required in order to propagate enough viral DNA for sequencing, it is possible that additional mutations could have arisen during limited in vitro propagation. However, based on the dramatic changes in allele frequencies in some cases a total reversion of allele frequency in late passage was observed (see below), we believe the polymorphic bases identified in this report were present in the plaque-purified populations. Similarly polymorphic bases have been identified in plaque purified isolates of three strains of the

Table 1
Vaccine strains and field isolates utilized in full genome comparisons.

Vaccines & isolates	Viral DNA sequencing	Origin	Genotype	Virulence
CEO Trachivax[a]	p1 CK[c]	Trachivax LT#67/2008[d]	IV	0[g]
CEO p1[a]	p2 CK	Fowl laryngotracheitis, 1991[e]	IV	0[h]
CEO p20[a]	p2 CK	Fowl laryngotracheitis, 1991 [e]	IV	+++[h]
63140[b]	P2 CK	Broiler isolate, GA. 2006	V	++++[i]
LT-IVAX[a]	p1 CK	LT-IVAX Serial #89364/ 2006[d]	II	0[g]
TCO p1[a]	p2 CK	LT-IVAX, 1991[f]	II	0[h]
TCO p20[a]	p2 CK	LT-IVAX, 1991[f]	II	+[h]
81658[b]	P2 CK	Broiler-breeder isolate, TX. 2010	III	++[j]
1874C5[b]	P2 CK	Broiler isolate	VI	++++[k]
USDA[b]	Unknown	Standard reference strain	I	++++[i]

[a] Genomes obtained in this study.
[b] Genomes obtained in Spatz et al. (2012).
[c] Passage in chicken kidney cells utilized for purification of viral DNA.
[d] Merck Animal Health, Summit, NJ, U.S.A.
[e] Vaccine from Vineland Laboratories Vineland, NJ, passed once (p1), or 20 times (p20) in chickens, followed by plaque purification in chicken kidney cells.
[f] Vaccine from American Scientific Laboratories, Schering Corp., Cream Reach, NJ, passed once (p1) and 20 consecutive times (p20) in chickens, followed by plaque purification in chicken kidney cells.
[g] Rodríguez-Avila et al., (2008).
[h] Guy et al. (1991).
[i] Vagnozzi et al. (2012).
[j] Non-published.
[k] Oldoni et al. (2009).

porcine alphaherpesvirus pseudorabies. Interestingly a shift in allele frequency upon ten passages of one of these strains Becker was reported by Szpara et al. (2011). To address this, future ILTV studies will undoubtedly involve sequencing directly from tissues of chicken infected with sequentially passed vaccine inoculums.

DNA statistics and genomic organization

In order to determine genes associated with attenuation/virulence, this comparative genomics study has identified gross mutations and polymorphic bases that differed among the newly sequenced US vaccine strains (Trachivax®, passages of Fowl Laryngotracheitis®, LT-Ivax® and passages of LT-Ivax®) and sequences of both virulent and vaccine strains currently deposited within GenBank.

The nucleotide sequences of the GaHV-1 vaccines strains have been submitted to GenBank and their accession numbers are as follows: LT-Ivax® (JN580312), LT-Ivax® p1 (JN580315), LT-Ivax® p20 (JN580314), Trachivax® (JN580313), Fowl Laryngotracheitis® p1 (JN580317) and Fowl Laryngotracheitis® p20 (JN580316). The DNA sequencing statistics with respect to the number of reads and the fold coverage are presented in Table 2. On average there was a 37 fold coverage with the 454 data and a 5594 fold coverage using Illumina data. Although the read lengths were considerably longer (average 270 bp) using the 454 pyroseqeuncing technology, the depth of coverage using the Illumina technology was over two logs greater. The use of both technologies allowed for the accurate resolution of homopolymer stretches (a shortcoming of 454 technology) and accurate *de novo* assembly of the genomes (a shortcoming of Illumina short read technology). The genome lengths (bp) of the GaHV-1 isolates are as follows: 155,465 (LT-Ivax® and LT-Ivax® p1); 150,335 (LT-Ivax® p20); 153,647 (Trachivax® and Fowl Laryngotracheitis® p20); and 153, 641 (Fowl Laryngotracheitis® p1). Unlike the class E-type genomic organization of the prototype alphaherpesvirus herpes simplex virus-1 (HSV-1) with inverted repeats bracketing both the unique long and unique short regions, the GaHV-1 genomes are organized as class D-type with inverted repeats flanking only the unique short region. The unique short regions of the two vaccine strains are identical in length (13,094). However, the unique long regions differ by 3340 base pairs (112,915 Trachivax® vs 109,575 LT-Ivax®). In order to investigate these differences a multiple sequence alignment (Fig. 1) of sequenced GaHV-1 genomes was generated using MAFFT (Katoh et al., 2005). The differences in the lengths of the unique long regions are attributed to 3337 bp deletions at the 5′ end of the genomes (Fig. 1B) and a three nucleotide deletion within the U_L37 gene of LT-Ivax and its passages. This large 3.3 kb deletion was also present within the genomes of the USDA and 81658, suggesting a common lineage of these virulent strains with the TCO LT-Ivax® vaccine strains.

Sequence length differences in the repeat regions also vary among the strains as well as between chicken passages (p1 and p20) of the TCO LT-Ivax® vaccine. The inverted repeats of all newly sequenced CEO genomes (Fig. 1C) are 13,819 bp in length, similar to the 13,833 bp length of the high passage LT-Ivax® (p20) inverted repeats. Interestingly, LT-Ivax® vaccine stock and LT-Ivax® p1 are significantly longer (16,398 bp) due to the presence of four copies of the 855 bp repeat within the hypervariable region (Fuchs et al., 2007) which is adjacent to the promoter region of the ICP4 gene.

Differences in the ICP4 genes of CEO and TCO genomes were identified through multiple sequence alignments. The genomes CEO clade members contain a single copy of the sequence gcggcccaagac (coordinates 145,200 to 145,211 in the USDA reference genome) per inverted repeat. Two tandem copies of this sequence are found within the ICP4 genes of TCO members. The significance of this duplication is unknown but may be useful in diagnostic assays.

Table 2
Sequencing statistics.

Vaccines & isolates	Total reads 454	Coverage	Total reads illumina	Coverage
CEO Trachivax®	55,575	8 ×	16,883,473	1,1582 ×
CEO p1	30,935	18 ×	3,539,234	2,428 ×
CEO p20	20,179	19 ×	11,444,063	8,014 ×
63140	28,100	20 ×	7,817,890	5,363 ×
LT-IVAX®	89,321	139 ×	6,210,920	4,284 ×
TCO p1	35,446	47 ×	9,096,173	6,339 ×
TCO p20	8,117	9 ×	1,367,246	980 ×
81658	ND	ND	8,370,909	5,759 ×

Phylogenetic analysis

The phylogenetic relationship among the newly sequenced genomes of US vaccine strains CEO (Trachivax® and Fowl Laryngotracheitis® p1) and TCO (LT-Ivax®), US virulent strains (63140, 81658, 1874C5, USDA reference), US CEO strains Laryngo-Vac® and LT Blen®, the European strain Serva, and Australian vaccine strains (SA2 and A20) and Australian virulent strains CL9 and ACC78 are depicted in Fig. 2. The evolutionary tree grouped the available GaHV-1 nucleotide sequences into four major clades (CEO, TCO, group V and AU) and suggests that the US vaccine strain TCO LT-Ivax® and CEO Trachivax®/Fowl Laryngotracheitis® form distinct evolutionary groups. Notably this tree is in agreement with the dendrogram that partitioned the US GaHV-1 isolates into nine genotypes based on cluster analysis of PCR-RFLP patterns (Oldoni and Garcia, 2007).

The CEO clade (in bold Fig. 2) includes the US CEO vaccine strains LT Blen®, Trachivax®, Laryngo-Vac®, Fowl Laryngotracheitis®, the US virulent group V strain 63,140, the European Serva vaccine and the virulent Australian natural recombinant ACC78. Interestingly, CEO clade members from the US mainly originated from the Hudson strain (Beaudette and Hudson, 1933). These include Trachivax®, Fowl Laryngotracheitis® and LT Blen®. Only one (Laryngo-Vac®) originated from the Cover strain. Based on the phylogenetic grouping and the timeline of isolation, the Cover strain (circa 1958) most likely originated from the Hudson strain (circa 1933).

The TCO clade includes the virulent USDA reference strain and strain 81658 which was isolated from a breeder flock where TCO-vaccinated males were mixed with non-vaccinated birds. This may suggest that strain 81658 is a vaccinal revertant. The phylogenetic tree also suggests that TCO strains either originated from the USDA reference strain or from a common ancestor. Notably both the USDA and 81658 strains contain the signature 3.3 kb deletion at the 5′ ends of their genomes (Fig. 2C).

The Australian vaccine strains (SA2 and A20) and Australian virulent strain CL9 are distantly related to the other GaHV-1 strains, as is 1874C5 strain, and forms the new Australian (AU) clade. Most interestingly is the placement of virulent Australian strains ACC78 outside of the Australian clade and within the CEO clade. Similarity plots (Lee et al., 2012) of the sequences of ACC78 and CL9 genomes with those of Serva, A20, and SA2 indicate that both genomes contain sequences derived from the Serva genomes. These plots also suggest virulent CL9 contain sequences derived from A20 and these sequences are largely responsible for its placement within the Australian clade. The placement of virulent ACC78 within the CEO clade is due to shared sequences with the Serva strain. Because similarity plots could not defined whether ACC78 originated from SA2 or A20 – the recombinant

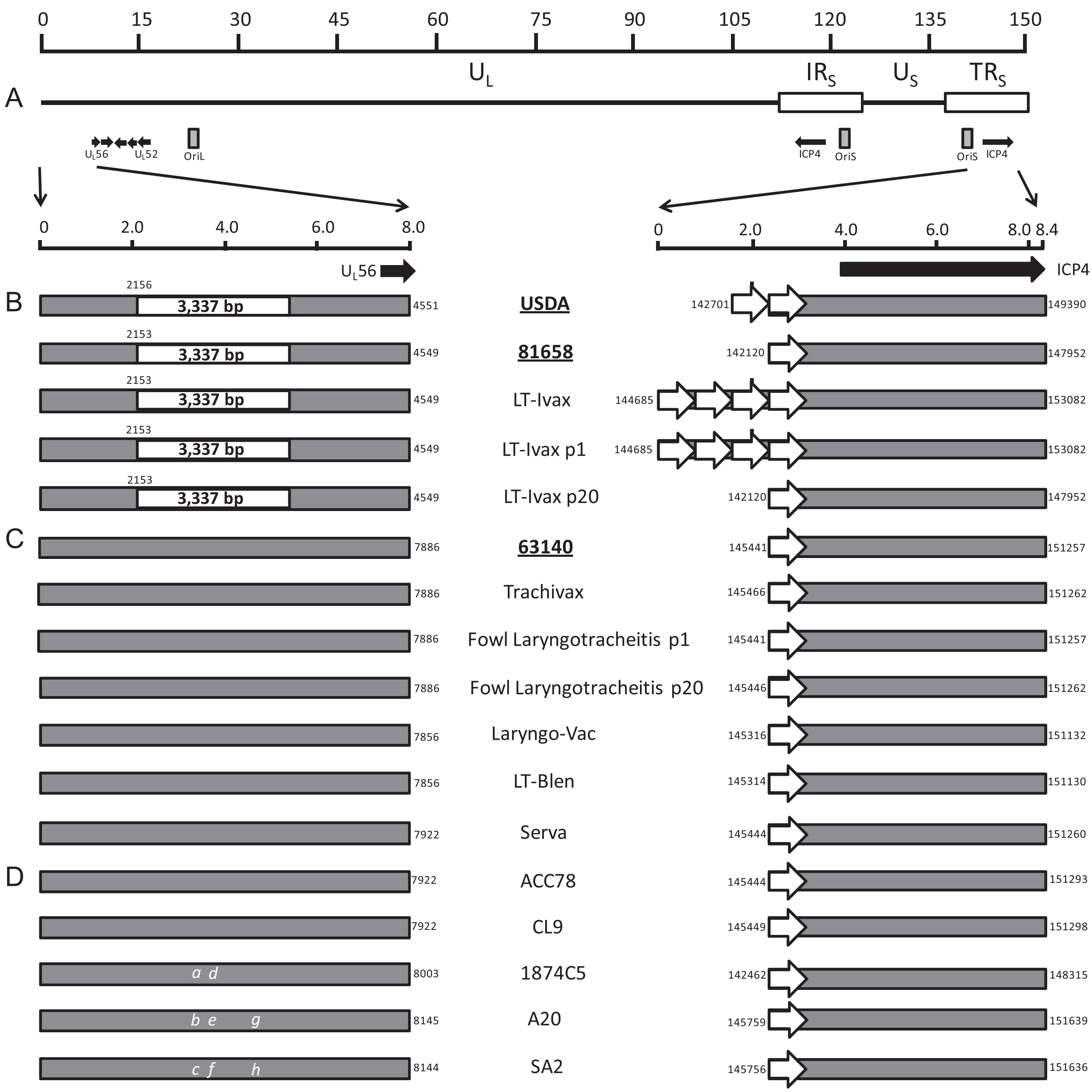

Fig. 1. The genomic location of the major deletions at the termini of the *Gallid herpesvirus* 1 genomes. (A) The typical GaHV-1 genome with the unique long (U_L) and unique short (U_S) which is surrounded by repeat regions; internal repeat short (IR_S) and terminal repeat short (TR_S). Black arrows represent selected ORFs and grey boxes denote origins of replication. Two regions at the termini of the GaHV-1 genomes containing gross genetic alterations were enlarged. (B) The termini of GaHV-1 strains USDA, 81648, LT-Ivax®, LT-Ivax® p1 and LT-Ivax® p20 (TCO clade members) showing the 3337 bp deletion at the 5′ termini and multiple copies (white arrows) of the 855 bp in the hypervariable region within the promoter of ICP4. The names of the virulent strains are in bold and underlined. (C) The termini of GaHV-1 strains 63140, Trachivax, Fowl Laryngotracheitis® p1, Fowl Laryngotracheitis® p20, Serva, Laryngo-Vac and LT-Blen (CEO clade members) showing the single copy (white arrows) of the 855 bp in the hypervariable region within the promoter of ICP4. (D) The termini of GaHV-1 strains of Australian strains ACC78, CL9, A20 and SA2; and US strain 1874C5 showing the 5′ termini containing insertions (*a*–*h*) and single copy (white arrows) of the 855 bp in the hypervariable region within the promoter of ICP4. The lengths and map coordinates for the insertions are as follows: *a*, 100 bp, 3263–3362; *b*, 193 bp, 3283–3475; *c*, 192 bp, 3283–3474; *d*, 20 bp, 3554–3573; *e*, 20 bp, 3667–3686; *f*, 20 bp, 3666–3685; *g*, 33 bp, 4655–4687; *h*, 33 bp, 4654–4686.

region was identical to both SA2 and A20 – the origin of its virulent component is unknown at this time.

Virulent versus vaccine

In the composite genome generated by Thureen and Keeler (2006) 80 ORFs were annotated based on similarities to the translation products of other herpesviruses genes. When comparing the genomes of the US CEO and TCO clade isolates, deletions were identified in only three genes (ORF C, U_L37 and ICP4). These deletions were all in frame and are unlikely to affect the protein's function. In contrast a point mutation was identified in the ORF C genes of some members of the TCO clade. This Iltovirus specific gene encodes a polypeptide of 334 amino acids with two protein products (30 and 38 kDa) as detected on Western Blots (Veits et al., 2003). This 334 amino acids protein is predicted for the ORF C genes of US GaHV-1 virulent isolates 63140, 1874C5, US CEO vaccine strains (Trachivax®, Fowl Laryngotracheitis® p1, Fowl Laryngotracheitis® p20, LT Blen®, Laryngo-Vac®) and European CEO Serva vaccine strain. However, due to a C→T mutation that introduces a TAA (ochre) stop codon, a

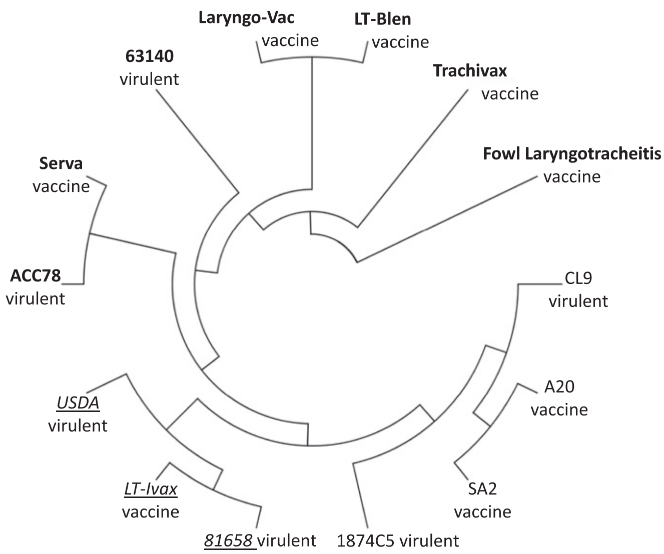

Fig. 2. Dendogram of 12 *Gallid herpesvirus* 1 strains. The tree was generated from a multiple alignment of whole genome sequences (unique long, internal repeat short and unique short) using the web-based MAFFT program. The tree was drawn using Tree Explorer of the phylogenetic and molecular evolutionary program MEGA version 4.0.2. The phylogenetic tree was constructed using complete genomes from GenBank for 81658 (JN542535), 1874C5 (JN542533) 63140/C/08/BR (JN542536) USDA reference strain (JN542534), SA2 (JN596962), A20 (JN596963), Laryngo-Vac® (JQ083494) LT-Blen® (JQ083493), Serva (HQ630064), CL9 (JN804827), ACC78 (JN804826) and genome sequences generated in this study for LT-Ivax® (JN580312), Trachivax® (JN580313), and Fowl Laryngotracheitis® (JN580317). Members of the CEO clade are in bold. Members of the TCO clade are italicized and underlined.

truncated 303 amino acid polypeptide is predicted for ORF C genes in all TCO genomes sequenced (LT-Ivax®, LT-Ivax®p1, LT-Ivax®p20). Interestingly this truncated protein was also predicted in the ORFC gene of virulent isolate 81658, a strain that originated from a flock experiencing severe ILT clinical signs after the introduction of cocks vaccinated with the LT-Ivax® vaccine. Preliminary data indicate that strain 81658 showed moderate virulence in experimentally infected broilers (data not shown).

Although both the LT-Ivax® and 81658 genomes contain the common mutation responsible for the ORF C truncation, minor differences do exist between the genomes of 81658 and LT-Ivax® p1. Only five nucleotides differed between the genomes of LT-Ivax® p1 and that of the virulent 81658 isolate. Specifically an intergenic T/G mutation mapped between the 3′ ends of U$_L$40 and U$_L$41 at nucleotide positions 68,481 for both LT-Ivax® and 81658, respectively. Of the four intragenic mutations identified two mapped within the unique long region and two within the repeat short region. A synonymous mutation (C/T at position 60,957) mapped within the U$_L$37 gene. Perhaps of more significance a T/C substitution at position 109,115 was identified within the intron of Iltovirus-specific gene UL-1. Only one non-synonymous mutation Met136Ile was identified which mapped within the diploid U$_S$10 genes (Iso136 in 81648) at positions 124,169/121,604 (G/A) and 140,872/138,307 (C/T) for LT-Ivax® and 81658, respectively. These U$_S$10 mutations (ATA codon) were unique to the virulent 81658 isolate and not found in other TCO clade members. All other published GaHV-1 genomes contain the ATG (Met) codon at this position. Little is known of the function of the U$_S$10 gene in GaHV-1. This annotated U$_S$10 homologue has very low sequence identities at the amino acid level (31.3%, 29.8%, 28.6% and 28.4%) to known U$_S$10 homologues of other avian herpesviruses (GaHV-3, GaHV-2, DHV-1 and MeHV-1, respectively). Interestingly, in GaHV-1 the U$_S$10 gene product has been demonstrated to interact with SCA2, a member of lymphostromal cell membrane Ly-6 superfamily, which is involved in T cell differentiation and activation (Liu et al., 2003).

Besides these differences both the LT-Ivax® and virulent 81658 genomes contain identical deletions at their 5′ termini (3337 bp after nucleotide 2153; Fig. 1). Furthermore both LT-Ivax® p20 and 81658 contained a single copy of the 855 bp repeat element per inverted repeat, unlike the 4 copies/inverted repeat found in the genomes of LT-Ivax® p1 and LT-Ivax® vaccine stock. Notably, LT-Ivax® p20 and the virulent strains USDA and 81658, contained fewer copies of the 855 bp repeat then their attenuated counterparts. This may indicate that expansion of this region attenuates the virus perhaps in a fashion similar to the attenuating expansion observed in the 132 bp repeat region within the genome of Marek's disease virus (Ross et al., 1989; Silva and Witter, 1985). Although it may be attractive to suggest that virulence of TCO members could be related to the loss of the number of 855 bp repeats, other vaccine strains outside the TCO clade (Serva, SA2 and A20) contain only a single copy of the 855 repeat element. It is largely suspected that the Ile136 non-synonymous mutation in U$_S$10, which is exclusively found in the 81658 genome is responsible for its virulent phenotype. Because the 81658 genome is 99.99% identical to that of LT-Ivax® and given the similarities mentioned above, it is highly likely that 81658 is a TCO revertant.

If strain 81658 is likely a TCO revertant perhaps virulent strain 63140 is a CEO revertant? Phylogenetically, 63140 partitioned with the US CEO vaccine strains and the European Serva strain (Fig. 2), indicating a common lineage. Furthermore, strain 63140 was isolated from a non-vaccinated broiler flock located in northeast Georgia, US, a region with a long history of ILT outbreaks that are frequently controlled using CEO vaccinations. To address this question, the genome of 63140 was compared to the genome of all CEO clade members. In particular, a comparison between 63140 and CEO Fowl Laryngotracheitis® p1 revealed 40 base pair changes. Of these, 20 mutations within 17 genes (ORF C, ORF E, ORF F, ICP4, U$_L$5, U$_L$10, U$_L$17, U$_L$23, U$_L$26, U$_L$27, U$_L$28, U$_L$36, U$_L$38, U$_L$39, U$_L$54, U$_S$5 and U$_S$8) were identified as intragenic (Table 3). However, the SNPs (codons in bold Table 3) found in the genes ICP4, U$_L$54, U$_L$5, U$_L$38 and U$_S$8 within the 63140 genome were also found in the genomes of other CEO vaccine strains. Discarding mutations found in these five genes, the remaining 12 genes may likely encode potential virulence factors. Based on the phylogenetic partitioning of the 63140 genome along the CEO branch and the relatively low number of mutations that were exclusive to the 63140 genome, it is postulated that 63140 evolved from CEO-like strains that circulated in the field.

The virulent reference strain USDA partitioned along with TCO vaccine LT-Ivax® and virulent 81658 strains. The genome of this reference strain differs from LT-Ivax® p1 by 111 SNPs and contains 21 non-synonymous mutations distributed in 12 genes (ORF C, U$_L$0, U$_L$-1, U$_L$5, U$_L$27, U$_L$28, U$_L$29, U$_L$37, U$_L$39, U$_L$46, U$_L$47 and ICP4 (Table 4). However, the GCA codon encoding Ala391 within the U$_L$5 genes of the USDA strain is also in the genomes of LT-Ivax® p20 and LT-Ivax® vaccine stock. Discarding this gene the list is reduced to 11 genes that are likely to encode virulence factors.

The US strain 1874C5 was phlylogenetically distinct from all other US strains. Twenty exclusive SNPs (Supplement Table 1) the majority of which were in two genes, U$_L$30 and ICP4 were catalogued. Because of its unique partitioning and the fact that all other sequenced virulent strains are phylogenetically related to vaccine strains, 1874C5 is unlikely to be a vaccinal revertant.

A difficult aspect in the identification of mutations related to virulence is the lack of a consensus found among all virulent isolates (USDA, 63140, 81658 and 1874C5). Mutations seem to be genome specific and most often fail to group among similar strain's pathotypes. A comparison between the virulent genes

Table 3
Genes containing non-synonymous mutations that differ among vaccine strain CEO p1 and virulent isolate 63140.

	Gene	AA change	AA position	Trachivax	CEO p1	CEO p20	63140	Laryngo-Vac	LT-Blen	Serva
1	ORF C	del-Asp	67	–	–	–	**gat**	–	–	–
2	ORF E	Gly-Ala	133	ggc	ggc	Ggc	**gcc**	ggc	ggc	ggc
3	ORF F	Thr-Ser	78	tca	tac	Tca	**aca**	tca	tca	tca
4	ICP4	Ala-Thr	1152	act	**gct**	**gct**	act	act	act	act
5	U$_L$54	Met-Ile	479	atg	atg	atg	**ata**	ata	atg	ata
6	U$_L$23	Arg-Trp	97	cgg	cgg	cgg	**tgg**	cgg	cgg	cgg
7	U$_L$39	Asp-Asn	20	gac	gac	gac	**aac**	gac	gac	gac
8	U$_L$36	Ser-Gly	359	agc	agc	agc	**ggc**	agc	agc	agc
9		Arg-His	1347	cgc	cgc	cgc	**cac**	cgc	cgc	cgc
10	U$_L$5	Lys-Glu	343	aaa	aaa	**gaa**	**gaa**	aaa	aaa	aaa
11	U$_L$26	Gln-Arg	179	cag	cag	cag	**cgg**	cag	cag	cag
12	U$_L$38	Val-Ala	290	gcc	**gtc**	gcc	gcc	gcc	gcc	gcc
13	U$_L$28	Asp-Gly	735	gac	gac	gac	**ggc**	gac	gac	gac
14	U$_L$17	Ala-Thr	320	gca	gca	gca	**aca**	gca	gca	gca
15	U$_L$27	Arg-His	496	cgc	cgc	cgc	**cac**	cgc	cgc	cgc
16		Ile-Thr	644	ata	ata	ata	**aca**	ata	ata	ata
17		Glu-del	809	gag	gag	gag	–	gag	gag	gag
18	U$_L$10	Thr-Ala	42	acc	acc	acc	**gcc**	acc	acc	acc
19	U$_S$5	Ala-Thr	340	gcc	gcc	gcc	**acc**	gcc	gcc	gcc
20	U$_S$8	Lys-Arg	210	aaa	aaa	aaa	**aga**	**aga**	aaa	aaa

Codons in bold are there for clarity.

Table 4
Genes containing non-synonymous mutations that differ between TCO p1 and virulent USDA reference strain.

	Gene	AA change	AA position	LT-Ivax	TCO p1	TCO p20	USDA	81658
1	ORF C	Gln–Stop	304	taa	taa	taa	**caa**	taa
2	U$_L$0	Pro–Ser	153	cca	cca	cca	**tca**	cca
3		Thr–Ala	378	aca	aca	aca	**gca**	aca
4	U$_L$(-1)	Ala–Val	252	gca	gca	gca	**gta**	gca
5	ICP4	Ala-Pro	199	gcc	gcc	gcc	**ccc**	gcc
6		Asp–Asn	747	aac	aac	aac	**gac**	aac
7		Asp-Gly	1333	gac	gac	gac	**ggc**	gac
8	U$_L$39	Gly-Cys	263	ggc	ggc	ggc	**tgc**	ggc
9		Ala-Thr	349	gca	gca	gca	**aca**	gca
10		Ser-Gly	530	agc	agc	agc	**ggc**	agc
11	U$_L$29	Asp-Gly	905	gac	gac	gac	**ggc**	gac
12	U$_L$5	Ala-Thr	391	gca	**aca**	gca	gca	**aca**
13	U$_L$37	Del-Leu	462	–	–	–	cta	–
14		Ile-Met	667	ata	ata	ata	**atg**	ata
15	U$_L$28	Glu–Gly	363	gaa	gaa	gaa	**gga**	gaa
16		Arg-Gln	374	cga	cga	cga	**caa**	cga
17		Ile–Thr	509	att	att	att	**act**	att
18	U$_L$46	Asp-Tyr	327	gac	gac	gac	**tac**	gac
19	sORF1	Asp-Gly	279	gac	gac	gac	**ggc**	gac
20	U$_L$27	Thr-Met	348	acg	acg	acg	**atg**	acg
21		Ile-Thr	44	ata	ata	ata	**aca**	ata

Codons in bold are there for clarity.

listed in Tables 3 and 4, reveals six genes (ORF C, U$_L$5, U$_L$27 (gB), U$_L$39, U$_L$28 and ICP4) that are common to both lists. Little is known about the role of the ORF C protein in GaHV-1 virulence. The helicase protein encoded by U$_L$5 has been identified as a virulence factor in HSV-1 by Biswas et al. (2008, 2009). By investigating resistance to helicase-primase inhibitors they discovered that mutants containing an Asn342Lys substitution grew slower with a moderate reduction in virulence compared to wild type. Strikingly, we have identified a Glu343Lys polymorphism between vaccine and virulent members of the CEO clade. It is postulated that Lys343 in the critical motif IV domain of U$_L$5 is an attenuating mutation and affects the viral DNA replication in CEO vaccine strains.

It was not surprising that mutations in the genes encoding glycoproteins (e.g. gB, gM, gJ and gE) were identified in this study since they are involved in binding of the virus to susceptible cells. However it was interesting that the gene most conserved among herpesviruses, U$_L$27 encoding glycoprotein B, contained a relatively large number of mutations. Four mutations (Arg496His, Ile644Thr, Met348Thr and ΔGlu809 were identified in the glycoprotein B genes. Interestingly the Thr644 mutation was found in all virulent strains of GaHV-1 including the newly sequenced Australian virulent isolates CL9 and ACC78 (Lee et al., 2012). Mutations in the genes encoding gB homologues of other herpesviruses have been reported (Goodman and Engel, 1991; Hutchinson et al., 1993) especially those involved in the syncytial (syn) phenotype and many strains of HSV-1 attribute their differences in virulence to mutations found in this gene. These differences likely affect receptor binding and immunogenicity since gB is a class III fusogen and major immunogen (Atanasiu et al., 2010).

Glycoproteins M, J and E encoded by the U$_L$10, U$_S$5 and U$_S$8 genes, respectively have also been implicated in virulence (Kaashoek et al., 1994; van Engelenburg et al., 1994; Zhang et al., 2009). Both gM and gE are essential for virus growth of GaHV-2 in cell cultures as reported by Tischer et al. (2002); however in GaHV-1 and in other alp will haherpesviruses (e.g HSV-1, PRV, BHV-1, EHV-1 and EHV-4), gM is inessential for lytic replication (Baines and Roizman, 1991; Fuchs and Mettenleiter, 1999; Kawabata et al., 2012; Konig et al., 2002; May et al., 2005). In GaHV-1 and GaHV-2, glycoprotein E in conjunction with glycoprotein I were found to be essential for growth in cultured cells (Devlin et al., 2006; Schumacher et al., 2001). However mutants containing deletions in gE have be described for vaccine strains of pseudorabies (e.g. Bartha) and vaccine strains of bovine herpesvirus 1 containing ΔgE deletions have been engineered by Lomniczi et al. (1984), Mettenleiter et al. (1985), Szpara et al. (2011), van Engelenburg et al. (1994)). Mundt have reported that Glycoprotein J of GaHV-1 has been reported to be inessential for lytic replication of GaHV-1 (Mundt et al., 2011) and recently ΔgJ mutants have been demonstrated to be attenuated and safe for *in ovo* inoculations to vaccinate against laryngotracheitis (unpublished results).

The large subunit of ribonucleotide reductase encoded by U$_L$39 has been reported to be a virulence factor of HSV-1, VZV (Brandt et al., 1991; Cameron et al., 1988; Heineman and Cohen, 1994) and GaHV-2 (Lucy Lee, personal communication). It is also within reason that mutations in the terminase protein U$_L$28 and the major transcriptional activator ICP4 could play a role in virulence since these proteins are involved in packaging/cleavage of the virus genome and activation of early/late gene expression, respectively (Yang and Baines, 2006; Yu and Weller, 1998). Notably some mutations (Thr509 and Asp742 within the U_L28 and ICP4 genes, respectively) have been identified in both the virulent 1874C5 and the USDA reference strains.

Sequence polymorphisms: Allele frequency

Two sequencing technologies were used to determine the genomic sequences presented in this paper. While the longer reads of 454 assisted initial assembly, the depth of coverage (>5000 fold) obtained by Illumina sequencing allowed for the identification of rare polymorphic bases in 8 genes (U_L37, U_L38, U_L41, U_L5 ICP4, U_S10, U_S5 and U_S8) out of 80 ORFs examined within the Trachivax®, LT-Ivax® and Fowl Laryngotracheitis® passage populations and the frequencies in which they occurred. Fluctuations in the allele frequencies were also identified at the origin of replication (OriS) and in microRNA ICP4:iltv-miR-16-3p within the ICP4 ORF. Similarly, Chandra et al. (2012) described mixed genome populations within vaccine preparations of Laryngo-Vac® and LT Blen.®

In this study we found that the Trachivax® and LT-Ivax® vaccines also contained mixed genome populations, but different genes were identified (U_L38, ICP4, U_S5 and U_S8 of CEO Trachivax® and U_L37, U_L5 and U_S10 of TCO LT-Ivax®) with grossly varying allele frequencies in each of the vaccine populations. Most of the codons that varied within a population encoded non-synonymous amino acid substitutions (Table 5). Only two genes U_L37 in TCO populations (LT-Ivax, LT-Ivax® p1 and LT-Ivax® p20) and U_S5 in passage populations of Fowl Laryngotracheitis® (CEO) contained codons encoding synonymous substitutions for glutamine and isoleucine, respectively (data not shown).

Table 5
Relevant allele frequencies for non-synonymous AA substitutions for Trachivax, LT-Ivax and passages in chickens of CEO (Fowl laryngotracheitis) and TCO (LT-Ivax) vaccines.

Isolate	Gene	Position	Allele 1	Allele 2	AA1	AA2	Allele 1*f*	Allele 2*f*
Trachivax	U_L38	290	gtc	gcc	Val	Ala	14.52	85.48
LT-Ivax							0.06	99.94
CEO p1							99.83	0.17
CEO p20							0.04	99.96
TCO p1							0.08	99.92
TCO p20							0.00	100.00
Trachivax	U_L41	270	aca	ata	Thr	Ile	99.97	0.03
LT-Ivax							99.94	0.06
CEO p1							99.95	0.05
CEO p20							4.97	95.30
TCO p1							100.00	0.00
TCO p20							99.87	0.130
Trachivax	U_L5	391	gca	aca	Ala	Thr	99.97	0.03
LT-Ivax							60.10	39.90
CEO p1							99.95	0.05
CEO p20							99.95	0.05
TCO p1							0.08	99.92
TCO p20							97.83	2.17
Trachivax	ICP4	1152	act	gct	Ala	Thr	40.90	59.10
LT-Ivax							0.00	100
CEO p1							99.75	0.25
CEO p20							99.87	0.13
TCO p1							0.08	99.92
TCO p20							0.00	100
Trachivax	U_S10	136	atg	ata	Met	Ile	99.99	0.01
LT-Ivax							75.34	24.66
CEO p1							0.15	99.98
CEO p20							0.02	100.00
TCO p1							63.85	36.15
TCO p20							99.50	0.50
Trachivax	U_S8	210	aaa	aga	Lys	Arg	55.88	44.12
LT-Ivax							0.06	99.94
CEO p1							99.95	0.05
CEO p20							99.79	0.21
TCO p1							0.00	100
TCO p20							0.00	100

Low and high passage comparisons

Deep sequencing analysis showed that fluctuations in allele frequencies also occurred during passage in chickens. When the genomes of CEO Fowl Laryngotracheitis® p1 and p20 were analyzed only 6 differences were identified. The intragenic differences were synonymous substitutions of Iso259 in U_S5 encoding glycoprotein J, and two non-synonymous substitutions, Val290Ala in U_L38 and The270Ile in U_L41. Intergenic mutations were identified mainly in homopolymers of guanine within the promoters of U_L-1, U_S10 and ICP4. Polymorphic (A/G) bases within the "back to back" promoters of ICP4 and U_S10 (position 121,465 the genomes of CEO Fowl Laryngotracheitis® p1) and a cytosine homopolymer length difference at the 3′ termini of Sorf4/3 and U_S10 were also identified. Unlike passages of Fowl Laryngotracheitis® (CEO), no intergenic mutations were found between passages of TCO LT-Ivax®. The contributions of these types of mutations in virulence acquisition are difficult to assess *in silico*, but since they mainly occur in homopolymers it is likely that they do not play a significant role in virulence.

Of greater significance and interest were the dramatic changes in the allele frequencies within two genes (U_L38 and U_L41) in passage populations of CEO Fowl Laryngotracheitis®. By passage 20 a near total reversal of allele frequencies was noted for the non synonymous mutations in U_L38 and U_L41 (Table 5). The predominate codon (99.95%) encoding Thr270 in U_L41 of the CEO Fowl Laryngotracheitis® p1 population was only present in 4.97% of the population by passage 20. It is hypothesized that the Ile270 mutation in U_L41 (present in 95% of the p20 populations) is responsible for the robust gain in virulence following passage as reported by Guy et al. (1991). U_L41 encodes a ribonuclease commonly referred to as virus host shutoff (vhs) and we postulate that this mutation increases the activity of the vhs protein so that stimuli (e.g. dsRNA) for nonspecific host defense mechanisms (interferon production) are curtailed. It has been reported that HSV-1 mutants containing a deletion in the U_L41 gene are 5–20 fold more sensitive to interferon alpha and beta compared to the wild type strain, respectively (Suzutani et al., 2000). It is less likely that the dominate Ala290 mutation in U_L38 within p20 populations contributes to virulence since this mutation is also found in Trachivax® populations at a high frequency (85.5%).

The passage-associated gain in virulence of Fowl Laryngotracheitis® CEO strains are in stark contrast with those results observed with passages of the TCO vaccine LT-Ivax® in which conversion to virulence occurred at a much lower frequency; concurrently with the loss of three copies of the 855 base pair repeat per inverted repeat region and some changes in allele frequencies (Table 5). Even though the codons for Ala391 in U_L5 and Met136 in U_S10 were present in greater proportions in LT-Ivax® p20 populations, their contribution to virulence is doubtful due to the high proportion (60.10%) of the Ala391 codon in the U_L5 gene within the genome of LT-Ivax vaccine, and the high proportions of the codon for Met136 in U_S10 found in both passages (LT-Ivax® p1 and LT-Ivax® p20) (63.85% vs 99.5%). Furthermore, given that the Ile136 mutation in U_S10 was the only mutation that differentiated virulent 81658 from LT-Ivax®

p1 it is unlikely that the Met136 mutation plays a role in virulence.

Genetic differences and pathogenicity of the GaHV-1 strains and passages

During peer-review of this manuscript (Lee et al., 2012) provided convincing evidence that a virulent class 9 field virus (CL9) resulted from natural recombination of attenuated vaccine strains of Australian-origin (A20) and the European-origin Serva. To investigate the likelihood that U.S. virulent strains 63140 and 81658 resulted from a recombination event involving either LT-Ivax®, Fowl Laryngotracheitis® or Trachivax® whole-genome multiple alignments were generated in MAFFT Katoh et al. (2005) and used to produce similarity plots (data not shown) based on the methods of Lole et al. (1999). Positions containing gaps were stripped from the multiple alignment and percent identity was calculated over 6000 base pair sliding window with a step size of 20. Plots were generated from the resulting tables using R (Team, 2009). These plots show that the 63140 and 81658 are more closely related to CEO and TCO strains, respectively than those compared in the (Lee et al., 2012) paper, and there is no obvious recombination events based on visual assessment. This suggests that virulent strains 63,140 and 81658 arose through a mechanism other than homologous recombination between vaccine strains of CEO and TCO.

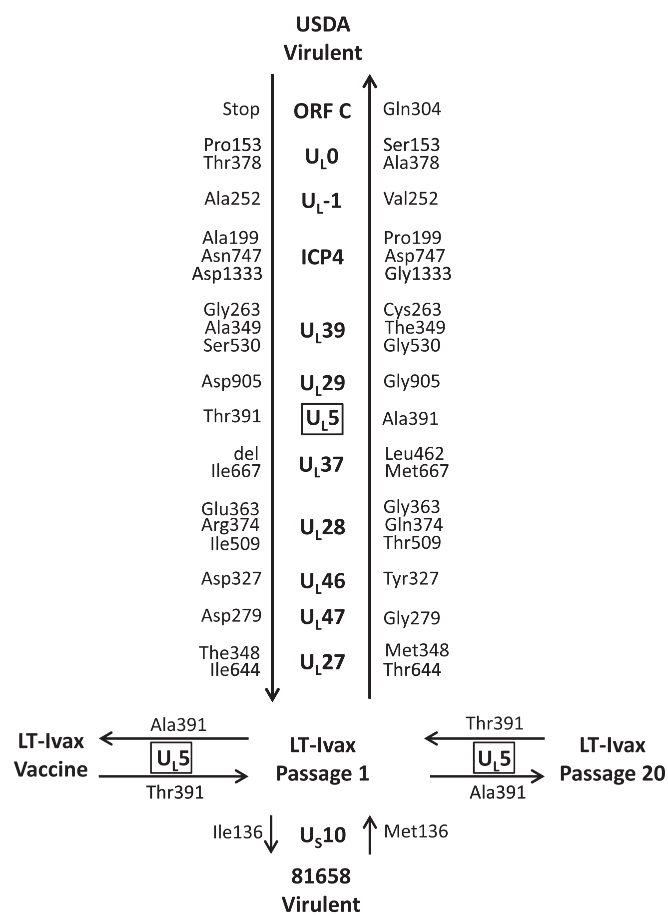

Fig. 3. Summary of amino acid changes among the TCO clade members USDA, 81658, LT-Ivax®, LT-Ivax® p1 and LT-Ivax® p20 involved in virulence/attenuation phenotypes. Arrows indicate the direction of the amino acid change for the phenotypes. The boxes indicate genes containing non-synonymous substitutions (corresponding to AA in bold in Table 4) that are found in the genomes of other GaHV-1 strains within the clade.

Summaries of the genes implicated in virulence/attenuation of TCO and CEO clade members based on our comparative genomic study are presented in Figs. 3 and 4. Overall few mutations were found in common among the two distinct lineages of ILT vaccines. Furthermore as reported in the study by Guy et al. (1991), gross differences in their phenotypes upon serial passages in birds suggest multiple genes are involved in reversion to virulence. High passage of TCO vaccine LT-Ivax resulted in only moderate gains in virulence. Genomic differences between plaque purified passages of LT-Ivax were identified only in the region containing the 855 bp repeats (Fig. 1). This correlation between virulence and the reductions in the number of copies of the 855 bp repeats appears to be TCO clade specific since single copies per inverted repeat were also identified in CEO clade members. Reversion to virulence of a TCO vaccine strain may involve (i) deletions in the 855 bp repeats to <2 copies per inverted repeats as seen with the virulent USDA TCO strain, (ii) the repair of the ORF C truncation and (iii) introduction of 21 mutations in 12 genes. Virulent strain 81658 appears to be a vaccinal revertant due to the isoleucine136 mutation within the U_S10, in spite of its retention of the ORF C truncation (Fig. 3).

Minor sequence differences were identified between the two Hudson strain derivatives Trachivax® and Fowl Laryngotracheitis® used in this study. They include a synonymous mutation for Gly212 in the U_L-1 gene and two non-synonymous mutations: Ala290Val in U_L38 and Thr1152Ala in ICP4 (Fig. 4). In comparing passages of plaque-purified Fowl Laryngotracheitis® rapid acquisition of virulence after 20 passages in birds is likely due to the Ile270 mutation in the virus host shutoff (vhs) protein encoded by U_L41 and probably does not involve the Ala290 mutation in U_L38 since this mutation is also found in the CEO vaccine strain Trachivax® and virulent strain 63140. CEO member strain 63140 is virulent because of 20 single nucleotide polymorphisms distributed in 17 genes.

Conclusions

Phylogenetic analysis indicates that CEO strains Trachivax®, Fowl Laryngotracheitis®, LT Blen®, Laryngo-Vac® and the European Serva are part of a clade that include the virulent strain 63140. Mutations in 12 genes (ORF C, ORF E, ORF F, U_S5 U_L17, U_L23, U_L26, U_L27, U_L28, U_L36, U_L39 and U_L10) were identified that were exclusively found within the genome of virulent 63140 relative to the genomes of all other CEO genomes. One additional gene U_L41 is likely to encode the virulence factor responsible for the rapid gain in virulence upon passage of the CEO strain Fowl Laryngotracheitis® in birds.

The TCO strain LT-Ivax® partitioned with the virulent strains 81658 and USDA reference and formed a unique clade distinct from the CEO clade. A mutation in ORF-C resulting in a predicted truncated polypeptide, was identified in vaccine strain LT-Ivax®. Five mutations were identified that differed among the genomes of LT-Ivax® and 81658; one of these (U_S10) is likely involved in its virulence. Mutations in 11 genes (ORF C, U_L0, U_L-1, U_L27, U_L28, U_L29, U_L37, U_L39, U_L46, U_L47 and ICP4) were exclusive for the USDA genome. No non-synonymous mutations exclusive for higher passages of TCO LT-Ivax® were identified. Moderate gains in virulence of higher passages of TCO LT-Ivax® are likely to involve deletions in the copy number of the 855 bp repeat within the ICP4 promoter.

In comparing the GaHV-1 genomes of virulent (63140, 81658, and USDA) and vaccine strains (Trachivax®, Fowl Laryngotracheitis®, LT Blen®, Laryngo-Vac®, Serva, SA2 and A20) mutations associated with attenuation/virulence were identified in four genes encoding the Ilto virus specific gene ORF C of unknown function, glycoprotein B

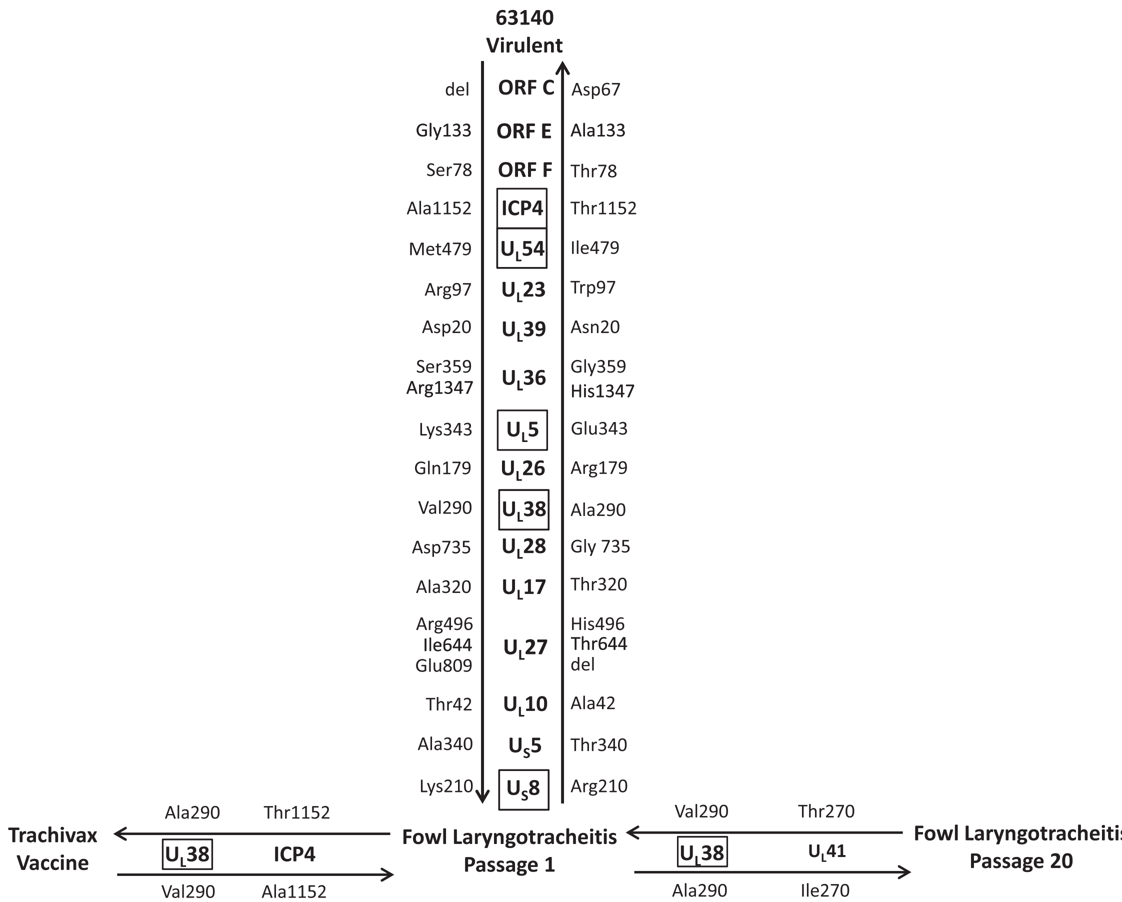

Fig. 4. Summary of amino acid changes among the CEO clade members 63140, Trachivax, Fowl Laryngotracheitis® p1 and Fowl Laryngotracheitis® p20 involved in virulence/attenuation phenotypes. Arrows indicate the direction of the amino acid change. The boxes indicate genes containing non-synonymous substitutions (corresponding to AA in bold in Table 3) that are found in the genomes of other GaHV-1 strains within the clade.

encoded by U_L27, cleavage/packaging protein encoded by U_L28 and ribonucleotide reductase encoded by U_L39.

Material and methods

Virus and cells

The GaHV-1 viruses utilized in this study were the chicken embryo origin (CEO) vaccine Trachivax® and Fowl Laryngotracheitis®, and the tissue culture origin (TCO) vaccine LT-Ivax® (Merck Animal Health, Summit, N.J. US). In order to isolate viral DNA, all viruses listed in Table 1 were propagated on confluent monolayers of chicken kidney (CK) cells using a MOI of 0.1 as described previously by Tripathy and Garcia (2008). The micrococcal nuclease/polyethylene glycol procedure (Volkening and Spatz, 2009) was used to isolate nucleocapsid DNA from the isolates listed in Table 1.

High-throughput DNA sequencing

Sequencing of the GaHV-1 vaccine genomes was accomplished using two sequencing technologies: 454 pyrosequencing and Illumina sequence-by-synthesis. Initially sequencing was performed on the Genome Sequencer 20 (GS20) system (454 Life Sciences) at the Georgia Genomics Facility (University of Georgia, Athens, GA) was used. To overcome limitations in 454 pyrosequencing related to homopolymer resolution and to fill remaining coverage gaps, all vaccine strains and the group III isolate 81658 were re-sequenced using the Illumina HiSeq 2000 sequencing platform running HCS v1.4.8 (Illumina Inc., San Diego, CA) at the Genome Services Lab at HudsonAlpha Institute for Biotechnology (Huntsville, Alabama).

For the initial 454 pyrosequencing, random libraries were constructed using 5.0 µg of nucleocapsid-purified DNA according to the methodology as described by Margulies et al. (2005). Single read genomic DNA libraries for Illumina sequencing were prepared from 5 µg of nucleocapsid purified genomic DNA that was fragmented by sonication to an average length of 200 bp. The samples were processed and indexed according to Illumina Library Creation kit's instructions, clustered on a cBot v1.4.36.0 using Illumina'sTruSeq PE Cluster Kit v2.0 and sequenced on one flowcell lane using a 200 cycle TruSeq SBS HS v2 kit. The clustered flow-cell was sequenced for 206 cycles, broken down into 3 separate reads. The first read was 100 cycles in length, followed by a six-cycle index read. Following the index read, paired end re-synthesis was performed using TruSeq PE Cluster Kit v2.0, which was then followed by another 100 cycles. Image analysis and base calling were performed using the standard Illumina Pipeline consisting of Real time Analysis (RTA) version v1.12.4.2 and Casava v1.8 with default settings.

GaHV-1 DNA sequence analysis

Pyrosequencing data was assembled from reads using GS De Novo Assembler (Roche, Indianapolis, IN), Sequencher (Gene Codes, Ann Arbor, MI) and the MIRA assembler v3.2.1 (Chevreux et al., 1999). Regions containing mononucleotide reiterations >6, ambiguities and gaps in the deep-sequencing data were resolved through Illumina re-sequencing and reassembly using MIRA. The complete genomic sequences of all the vaccine strains and their passage progenies therefore represent hybrid assembly from two sequencing technologies. After assembly of each sample, reads were re-mapped to the consensus sequence using BWA (http://dx.doi.org/10.1093/bioinformatics/btp324). A pileup file containing sequencing depth data was generated from the resulting SAM file using samtools (http://dx.doi.org/10.1093/bioinformatics/btp352). This file was parsed to generate base frequencies at each position in the alignment, ignoring individual read positions with base qual < 10 on the phred scale. Multiple sequence alignments of all eight genomes were generated using MAFFT (Katoh et al., 2005) in order identify SNPs at the consensus level. Only these consensus SNPs were evaluated at the read depth level. Each SNP position was examined and "positions of interest" were determined where, for any of the samples, two or more bases each made up ≥ 2% of the total depth (qual ≥ 10). In other words, at least one sample had a minority population ≥ 2%. In determining both quality and frequency cutoffs we took into consideration the typical Illumina error rate, which is generally found to be < 1% across reads.

In order to identify single nucleotide polymorphisms, synonymous and non-synonymous substitutions and insertions/deletion (indels) newly sequenced genomes were compared to all non-composite GaHV-1 genomic sequences in GenBank (81658-JN542535, 1874C5-JN542533, 63140/C/08/BR-JN542536, USDA-JN542534, SA2-JN596962, A20-JN596963, Laryngo-Vac®-JQ083494, LT Blen®–JQ083493, Serva-HQ630064, ACC78-JN804826 and CL9-JN804827 using multiple sequence alignments MAFFT (Katoh et al., 2005) and the Mega4 programs (Kumar et al., 2004). Short nucleotide alignments were performed using Multalin (Corpet, 1988). All genomic sequences were maintained and analyzed using Lasergene (DNASTAR, Madison, WI), NCBI Entrez, and other web-based tools. Homology searches were conducted using the NCBI program BLAST with default settings.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.02.007.

References

Andreasen Jr., J.R., Glisson, J.R., Goodwin, M.A., Resurreccion, R.S., Villegas, P., Brown, J., 1989. Studies of infectious laryngotracheitis vaccines: immunity in layers. Avian Dis. 33 (3), 524–530.

Atanasiu, D., Saw, W.T., Cohen, G.H., Eisenberg, R.J., 2010. Cascade of events governing cell–cell fusion induced by herpes simplex virus glycoproteins gD, gH/gL, and gB. J. Virol. 84 (23), 12292–12299.

Bagust, T.J., Johnson, M.A., 1995. Avian infectious laryngotracheitis: virus-host interactions in relation to prospects for eradication. Avian. Pathol. 24 (3), 373–391.

Baines, J.D., Roizman, B., 1991. The open reading frames UL3, UL4, UL10, and UL16 are dispensable for the replication of herpes simplex virus 1 in cell culture. J. Virol. 65 (2), 938–944.

Beaudette, F.R., Hudson, C.B., 1933. Newly recognized poultry disease. North Am. Vet. 14, 50.

Biswas, S., Miguel, R.N., Sukla, S., Field, H.J., 2009. A mutation in helicase motif IV of herpes simplex virus type 1 UL5 that results in reduced growth in vitro and lower virulence in a murine infection model is related to the predicted helicase structure. J. Gen. Virol. 90 (Pt 8), 1937–1942.

Biswas, S., Tiley, L.S., Zimmermann, H., Birkmann, A., Field, H.J., 2008. Mutations close to functional motif IV in HSV-1 UL5 helicase that confer resistance to HSV helicase–primase inhibitors, variously affect virus growth rate and pathogenicity. Antiviral Res. 80 (1), 81–85.

Brandly, C.A., Bushnell, L.D., 1934. A report of some investigations of ILT. Poult. Sci. 13, 212–217.

Brandt, C.R., Kintner, R.L., Pumfery, A.M., Visalli, R.J., Grau, D.R., 1991. The herpes simplex virus ribonucleotide reductase is required for ocular virulence. J. Gen. Virol. 72 (Pt 9), 2043–2049.

Cameron, J.M., McDougall, I., Marsden, H.S., Preston, V.G., Ryan, D.M., Subak-Sharpe, J.H., 1988. Ribonucleotide reductase encoded by herpes simplex virus is a determinant of the pathogenicity of the virus in mice and a valid antiviral target. J. Gen. Virol. 69 (Pt 10), 2607–2612.

Chandra, Y.G., Lee, J., Kong, B.W., 2012. Genome sequence comparison of two United States live attenuated vaccines of infectious laryngotracheitis virus (ILTV). Virus Genes 44 (3), 470–474.

Chevreux, B., Wetter, T., Suhai, S., 1999. Genome sequence assembly using trace signal and additional sequence information. Comp.Sci. Biol. GCB 1, 45–56.

Cochran, M.D., MacDonald, R.D. (1998). Recombinant Herpesvirus of Turkeys and Uses Thereof. Vol. 5,853,733. Syntro Corpoaration, USA.

Corpet, F., 1988. Multiple sequence alignment with hierarchical clustering. Nucleic. Acids Res. 16 (22), 10881–10890.

Cover, M.S., Benton, W.J., 1958. The biological variation of infectious laryngotracheitis virus. Avian Dis. 2, 375–383.

Creelan, J.L., Calvert, V.M., Graham, D.A., McCullough, S.J., 2006. Rapid detection and characterization from field cases of infectious laryngotracheitis virus by real-time polymerase chain reaction and restriction fragment length polymorphism. Avian Pathol. 35 (2), 173–179.

Davison, A.J., Eberle, R., Hayward, G.S., McGeoch, D.J., Minson, A.C., Pellet, P.E., Roizman, B., Studdert, M.J., Thiry, E., 2005a. Family herpesviridae. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Virus Taxonomy: Eight Report of the International Committee on Taxonony of Viruses. Academic Press, San Diego, CA, pp. 193–212.

Davison, S., Dufour-Zavala, L., García, M., Ghori, H., Hoerr, F., Hopkins, B., Smith, J., Waldrip, D., 2005b. Proceedings of the 109th Annual Meeting of the United States Animal Health Association, Hershey, PA, 580–603.

Devlin, J.M., Browning, G.F., Gilkerson, J.R., 2006. A glycoprotein I- and glycoprotein E-deficient mutant of infectious laryngotracheitis virus exhibits impaired cell-to-cell spread in cultured cells. Arch. Virol. 151 (7), 1281–1289.

Fuchs, W., Mettenleiter, T.C., 1999. DNA sequence of the UL6 to UL20 genes of infectious laryngotracheitis virus and characterization of the UL10 gene product as a nonglycosylated and nonessential virion protein. J. Gen. Virol. 80 (Pt 8), 2173–2182.

Fuchs, W., Veits, J., Helferich, D., Granzow, H., Teifke, J.P., Mettenleiter, T.C., 2007. Molecular biology of avian infectious laryngotracheitis virus. Vet. Res. 38 (2), 261–279.

Fulton, R.M., L., S.D., Will, M., 2000. Effect of route of vaccination on the prevention of infectious laryngotracheitis in commercial egg-laying chickens. Avian Dis. 4, 8–16.

Gelenczei, E., Marty, E., 1962. Strain stability and immunologic characterizatics of tissue-culture modified infectious laryngotracheitis virus. Avian Dis. 9, 44–56.

Goodman, J.L., Engel, J.P., 1991. Altered pathogenesis in herpes simplex virus type 1 infection due to a syncytial mutation mapping to the carboxy terminus of glycoprotein B. J. Virol. 65 (4), 1770–1778.

Guy, J.S., Bagust, T.J., 2003. Laryngotracheitis. In: Saif, Y.M., Barnes, H.J., Glisson, J.R., Fadly, A.M., McDougald, L.R., Swayne, D.E. (Eds.), Dis. Poult., 11th Ed. Iowa State Press, Ames, IA, pp. 121–134.

Guy, J.S., Barnes, H.J., Munger, L.L., Rose, L., 1989. Restriction endonuclease analysis of infectious laryngotracheitis viruses: comparison of modified-live vaccine viruses and North Carolina field isolates. Avian Dis. 33 (2), 316–323.

Guy, J.S., Barnes, H.J., Smith, L., 1991. Increased virulence of modified-live infectious laryngotracheitis vaccine virus following bird-to-bird passage. Avian Dis. 35 (2), 348–355.

Guy, J.S., Garcia, M., 2008. Infectious laryngotracheitis virus. In: Saif, Y.M., Glisson, J.R., Fadly, A.M., McDougald, L.R., Nolan, L.K., Swayne, D.E. (Eds.), Diseases of Poultry, 12th Ed. Wiley-Blackwell, Hoboken, NJ.

Han, M.G., Kim, S.J., 2001. Analysis of Korean strains of infectious laryngotracheitis virus by nucleotide sequences and restriction fragment length polymorphism. Vet Microbiol. 83 (4), 321–331.

Han, M.G., Kim, S.J., 2003. Efficacy of live virus vaccines against infectious laryngotracheitis assessed by polymerase chain reaction–restriction fragment length polymorphism. Avian Dis. 47 (2), 261–271.

Heineman, T.C., Cohen, J.I., 1994. Deletion of the varicella-zoster virus large subunit of ribonucleotide reductase impairs growth of virus in vitro. J. Virol. 68 (5), 3317–3323.

Hilbink, F.W., Oei, H.L., van Roozelaar, D.J., 1987. Virulence of five live vaccines against avian infectious laryngotracheitis and their immunogenicity and spread after eyedrop or spray application. Vet Q 9 (3), 215–225.

Hughes, C.S., Gaskell, R.M., Jones, R.C., Bradbury, J.M., Jordan, F.T., 1989. Effects of certain stress factors on the re-excretion of infectious laryngotracheitis virus from latently infected carrier birds. Res. Vet. Sci. 46 (2), 274–276.

Hutchinson, L., Graham, F.L., Cai, W., Debroy, C., Person, S., Johnson, D.C., 1993. Herpes simplex virus (HSV) glycoproteins B and K inhibit cell fusion induced by HSV syncytial mutants. Virology 196 (2), 514–531.

Kaashoek, M.J., Moerman, A., Madic, J., Rijsewijk, F.A., Quak, J., Gielkens, A.L., van Oirschot, J.T., 1994. A conventionally attenuated glycoprotein E-negative strain of bovine herpesvirus type 1 is an efficacious and safe vaccine. Vaccine 12 (5), 439–444.

Katoh, K., Kuma, K., Toh, H., Miyata, T., 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Res. 33 (2), 511–518.

Kawabata, A., Jasirwan, C., Yamanishi, K., Mori, Y., 2012. Human herpesvirus 6 glycoprotein M is essential for virus growth and requires glycoprotein N for its maturation. Virology 429 (1), 21–28.

Keeler Jr., C.L., Hazel, J.W., Hastings, J.E., Rosenberger, J.K., 1993. Restriction endonuclease analysis of Delmarva field isolates of infectious laryngotracheitis virus. Avian Dis. 37 (2), 418–426.

Keller, L.H., Benson, C.E., Davison, S., Eckroade, R.J., 1992. Differences among restriction endonuclease DNA fingerprints of Pennsylvania field isolates,

vaccine strains, and challenge strains of infectious laryngotracheitis virus. Avian Dis. 36 (3), 575–581.
Kirkpatrick, N.C., Mahmoudian, A., O'Rourke, D., Noormohammadia, A.H., 2006. Differentiation of infectious laryngotacheitis vius isolates by restriction fragment length polymorphis analysis of polymerase chain reaction products amplified from multiple genes. Avian Dis 50, 28–34.
Konig, P., Giesow, K., Keil, G.M., 2002. Glycoprotein M of bovine herpesvirus 1 (BHV-1) is nonessential for replication in cell culture and is involved in inhibition of bovine respiratory syncytial virus F protein induced syncytium formation in recombinant BHV-1 infected cells. Vet. Microbiol. 86 (1-2), 37–49.
Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5 (2), 150–163.
Lee, S.W., Devlin, J.M., Markham, J.F., Noormohammadi, A.H., Browning, G.F., Ficorilli, N.P., Hartley, C.A., Markham, P.F., 2011a. Comparative analysis of the complete genome sequences of two Australian origin live attenuated vaccines of infectious laryngotracheitis virus. Vaccine 29 (52), 9583–9587.
Lee, S.W., Markham, P.F., Coppo, M.J., Legione, A.R., Markham, J.F., Noormohammadi, A.H., Browning, G.F., Ficorilli, N., Hartley, C.A., Devlin, J.M., 2012. Attenuated vaccines can recombine to form virulent field viruses. Science 337 (6091), 188.
Lee, S.W., Markham, P.F., Markham, J.F., Petermann, I., Noormohammadi, A.H., Browning, G.F., Ficorilli, N.P., Hartley, C.A., Devlin, J.M., 2011b. First complete genome sequence of infectious laryngotracheitis virus. BMC Genomics 12, 197.
Liu, H.C., Niikura, M., Fulton, J.E., Cheng, H.H., 2003. Identification of chicken lymphocyte antigen 6 complex, locus E (LY6E, alias SCA2) as a putative Marek's disease resistance gene via a virus-host protein interaction screen. Cytogenet. Genome. Res. 102 (1-4), 304–308.
Lole, K.S., Bollinger, R.C., Paranjape, R.S., Gadkari, D., Kulkarni, S.S., Novak, N.G., Ingersoll, R., Sheppard, H.W., Ray, S.C., 1999. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J Virol. 73 (1), 152–160.
Lomniczi, B., Blankenship, M.L., Ben-Porat, T., 1984. Deletions in the genomes of pseudorabies virus vaccine strains and existence of four isomers of the genomes. J Virol. 49 (3), 970–979.
Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A., Berka, J., Braverman, M.S., Chen, Y.J., Chen, Z., Dewell, S.B., Du, L., Fierro, J.M., Gomes, X.V., Godwin, B.C., He, W., Helgesen, S., Ho, C.H., Irzyk, G.P., Jando, S.C., Alenquer, M.L., Jarvie, T.P., Jirage, K.B., Kim, J.B., Knight, J.R., Lanza, J.R., Leamon, J.H., Lefkowitz, S.M., Lei, M., Li, J., Lohman, K.L., Lu, H., Makhijani, V.B., McDade, K.E., McKenna, M.P., Myers, E.W., Nickerson, E., Nobile, J.R., Plant, R., Puc, B.P., Ronan, M.T., Roth, G.T., Sarkis, G.J., Simons, J.F., Simpson, J.W., Srinivasan, M., Tartaro, K.R., Tomasz, A., Vogt, K.A., Volkmer, G.A., Wang, S.H., Wang, Y., Weiner, M.P., Yu, P., Begley, R.F., Rothberg, J.M., 2005. Genome sequencing in microfabricated high-density picolitre reactors. Nature 437 (7057), 376–380.
May, H.G., Tittsler, R.P., 1925. Tracheolaryngitis in poultry. J. Am.Vet. Med. Assoc. 67, 229–231.
May, J.S., Colaco, S., Stevenson, P.G., 2005. Glycoprotein M is an essential lytic replication protein of the murine gammaherpesvirus 68. J. Virol. 79 (6), 3459–3467.
Mettenleiter, T.C., Lukacs, N., Rziha, H.J., 1985. Pseudorabies virus avirulent strains fail to express a major glycoprotein. J. Virol. 56 (1), 307–311.
Mundt, A., Mundt, E., Hogan, R.J., Garcia, M., 2011. Glycoprotein J of infectious laryngotracheitis virus is required for efficient egress of infectious virions from cells. J. Gen. Virol. 92 (Pt 11), 2586–2589.
Neff, C.J., Sudler, C., Hoop, R.K., 2008. Characterisation of western European field isolates of avian infectious laryngotracheitis virus by restriction fragment length polymorphisms and sequencing. Avian Dis. 52, 278–283.
Ojkic, D., Swinton, J., Vallieres, M., Martin, E., Shapiro, J., Sanei, B., Binnington, B., 2006. Characterization of field isolates of infectious laryngotracheitis virus from Ontario. Avian Pathol. 35 (4), 286–292.
Oldoni, I., Garcia, M., 2007. Characterization of infectious laryngotracheitis virus isolates from the US by polymerase chain reaction and restriction fragment length polymorphism of multiple genome regions. Avian Pathol. 36 (2), 167–176.
Oldoni, I., Rodriguez-Avila, A., Riblet, S., Garcia, M., 2008. Characterization of infectious laryngotracheitis virus (ILTV) isolates from commercial poultry by polymerase chain reaction and restriction fragment length polymorphisms (PCR-RFLP). Avian Dis. 52, 59–63.
Oldoni, I., Rodriguez-Avila, A., Riblet, S.M., Zavala, G., García, M., 2009. Pathogenicity and Growth Characteristics of Infectious Laryngotracheitis Virus (ILTV) Isolates from United States. Avian Pathology 38, 47–53.
Rodríguez-Avila, A., Oldoni, I., Riblet, S., García., M., 2007. Replication and transmission of live-attenuated Infectiouslaryngotracheitis virus (ILTV) vaccines. Avian Dis. 51, 905–911.
Rodríguez-Avila, A., Oldoni, I., Riblet, S.M., García, M., 2008. Evaluation of the Protection Elicited by Direct and Indirect Exposure to Live Attenuated Infectious Laryngotracheitis Virus (ILTV) Vaccines Against a Recent Challenge Strain from the United States. Avian Pathology 37, 287–292.
Ross, L.J., Sanderson, M., Scott, S.D., Binns, M.M., Doel, T., Milne, B., 1989. Nucleotide sequence and characterization of the Marek's disease virus homologue of glycoprotein B of herpes simplex virus. J. Gen. Virol. 70 (Pt 7), 1789–1804.
Sadeghi, M., Bozorgemehrifard, M.H., Keyvanfar, H., Momtaz, H., Shooshtari, A., Charkhkar, S., 2011. Differentiation of field isolates and vaccine strains of infectious laryngotracheitis virus by DNA sequencing. Afr. J. Microbiol. Res. 5, 4112–4117.
Samberg, Y., Aronovici, I., 1969. The development of a vaccine against avian infectious laryngotracheitis. I. Modification of a laryngotracheitis virus. Refu. Vet 26, 54–59.
Schumacher, D., Tischer, B.K., Reddy, S.M., Osterrieder, N., 2001. Glycoproteins E and I of Marek's disease virus serotype 1 are essential for virus growth in cultured cells. J. Virol. 75 (23), 11307–11318.
Silva, R.F., Witter, R.L., 1985. Genomic expansion of Marek's disease virus DNA is associated with serial in vitro passage. J. Virol. 54 (3), 690–696.
Spatz, S.J., Volkening, J.D., Keeler, C.L., Kutish, G.F., Riblet, S.M., Boettger, C.M., Clark, K.F., Zsak, L., Afonso, C.L., Mundt, E.S., Rock, D.L., Garcia, M., 2012. Comparative full genome analysis of four infectious laryngotracheitis virus (*Gallid herpesvirus*-1) virulent isolates from the United States. Virus Genes. 44 (2), 273–285.
Suzutani, T., Nagamine, M., Shibaki, T., Ogasawara, M., Yoshida, I., Daikoku, T., Nishiyama, Y., Azuma, M., 2000. The role of the UL41 gene of herpes simplex virus type 1 in evasion of non-specific host defence mechanisms during primary infection. J. Gen. Virol. 81 (Pt 7), 1763–1771.
Szpara, M.L., Tafuri, Y.R., Parsons, L., Shamim, S.R., Verstrepen, K.J., Legendre, M., Enquist, L.W., 2011. A wide extent of inter-strain diversity in virulent and vaccine strains of alphaherpesviruses. PLoS Pathol. 7 (10), e1002282.
Team, R.D.C. (2009). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. Vienna, Austria.
Thureen, D.R., Keeler Jr., C.L., 2006. Psittacid herpesvirus 1 and infectious laryngotracheitis virus: comparative genome sequence analysis of two avian alphaherpesviruses. J. Virol. 80 (16), 7863–7872.
Tischer, B.K., Schumacher, D., Messerle, M., Wagner, M., Osterrieder, N., 2002. The products of the UL10 (gM) and the UL49.5 genes of Marek's disease virus serotype 1 are essential for virus growth in cultured cells. J. Gen. Virol. 83 (Pt 5), 997–1003.
Tong, G.Z., Zhang, S.J., Wang, L., Qiu, H.J., Wang, Y.F., Wang, M., 2001. Protection of chickens from infectious laryngotracheitis with a recombinant fowlpox virus expressing glycoprotein B of infectious laryngotracheitis virus. Avian Pathol. 30 (2), 143–148.
Tripathy, D.N., Garcia, M., 2008. Laryngotracheitis. In: Swayne, D.E., Glisson, J.R., Jackwood, M.W., Pearson, J.E., Reed, W.M. (Eds.), Isolation and Identification of Avian Pathogens, 5th Ed. University of Pennsylvania, New Bolton Center, PA, pp. 94–98.
Vagnozzi, A., Zavala, G., Riblet, S.M., Mundt, A., García, M., 2012. Protection induced by Infectious laryngotracheits virus (ILTV) Live-attenuated and Recombinant Viral Vector Vaccines in Broilers. Avian Pathology 41, 21–31.
van Engelenburg, F.A., Kaashoek, M.J., Rijsewijk, F.A., van den Burg, L., Moerman, A., Gielkens, A.L., van Oirschot, J.T., 1994. A glycoprotein E deletion mutant of bovine herpesvirus 1 is avirulent in calves. J. Gen. Virol. 75 (Pt 9), 2311–2318.
Veits, J., Mettenleiter, T.C., Fuchs, W., 2003. Five unique open reading frames of infectious laryngotracheitis virus are expressed during infection but are dispensable for virus replication in cell culture. J. Gen. Virol. 84 (Pt 6), 1415–1425.
Volkening, J.D., Spatz, S.J., 2009. Purification of DNA from the cell-associated herpesvirus Marek's disease virus for 454 pyrosequencing using micrococcal nuclease digestion and polyethylene glycol precipitation. J. Virol. Methods. 157 (1), 55–61.
Yang, K., Baines, J.D., 2006. The putative terminase subunit of herpes simplex virus 1 encoded by UL28 is necessary and sufficient to mediate interaction between pUL15 and pUL33. J. Virol. 80 (12), 5733–5739.
Yu, D., Weller, S.K., 1998. Herpes simplex virus type 1 cleavage and packaging proteins UL15 and UL28 are associated with B but not C capsids during packaging. J. Virol 72 (9), 7428–7439.
Zhang, J., Nagel, C.H., Sodeik, B., Lippe, R., 2009. Early, active, and specific localization of herpes simplex virus type 1 g to nuclear membranes. J. Virol. **83** (24), 12984–12997.